SEROLOGIC TESTS HAVE BEEN DEVELOPED WITH AN EMPHASIS ON THE DETECTION OF ANTIBODIES TO Fasciola gigantica, was expressed in Escherichia coli as a calmodulin binding peptide fusion protein with a molecular mass of approximately 35 kDa. The recombinant cathepsin L1 (rCTL1) was tested for its antigenic potential in a cystatin capture enzyme-linked immunosorbent assay (ELISA) to diagnose human fascioliasis. The ELISA plates were sensitized with chicken egg cystatin and incubated with bacterial lysates containing the recombinant protein before the standard ELISA procedures were performed. Analysis of the sera of 13 patients infected with F. gigantica (group 1), 204 patients with other parasitic infections (group 2), 32 cholangiocarcinoma patients (group 3), and 42 healthy controls (group 4) showed that the sensitivity, specificity, accuracy, positive predictive value, and negative predictive value of this ELISA using rCTL1 were 100%, 98.92%, 98.97%, 81.25%, and 100%, respectively. These results indicate that this assay has high sensitivity and specificity in the diagnosis of human fascioliasis. In addition, we have produced sufficient amounts of antigen for use in diagnosis.

INTRODUCTION

Fascioliasis is a disease caused by liver flukes of the genus Fasciola, of which F. hepatica and F. gigantica are the most common representatives. The disease is recognized as an important infectious condition by the World Health Organization and an estimated 17 million peoples are infected worldwide. Although F. hepatica has a worldwide distribution but predominates in temperate climates, and F. gigantica is also found in the tropical regions of Asia and Africa. The coprodagnosis of human fascioliasis is often unreliable because the eggs of the parasite are not found during the prepatent period. Even at later times, eggs are only intermittently released. Serologic diagnosis is preferred, particularly since antibodies to Fasciola can be detected as early as two weeks after infection and can facilitate early treatment before irreparable damage to the liver occurs.

A specific 27-kD antigen from F. gigantica (FG27) excretory-secretory products was purified and used as specific and sensitive antigen for the diagnosis of human fascioliasis. Recently, we partially sequenced the FG27 antigen and determined the sequence of the 20 amino acids from the N- to the C-terminus (Tantrawatpan C and others, unpublished data). The sequence data proved to be homologous with the deduced amino acid sequence of F. hepatica cathepsin L1 (GenBank accession number AF125866) and cathepsin L1-B (GenBank accession number AF239264) at positions 261 to 280, as well as with cathepsin L1-E (GenBank accession number AF239267) at positions 154 to 173.

Several serologic tests have been developed with an emphasis on the detection of antibodies to F. hepatica cathepsin L1. In the present study, we produced the recombinant F. gigantica cathepsin L1 (rCTL1) in Escherichia coli and examined the potential use of rCTL1 as a diagnostic reagent for human fascioliasis based on a cystatin capture enzyme-linked immunosorbent assay (ELISA). The method is relatively simple and does not require chromatographic purification of rCTL1 from a bacterial lysate of a recombinant clone.
stranded cDNA from isolated mRNA was carried out using the Marathon™ cDNA amplification kit (Clontech, Palo Alto, CA) according to the manufacturer’s instructions. After adapter ligation with the Marathon cDNA adapter, the 5’- and 3’-RACE PCRs were carried out using gene specific primers (GSPs) and the adapter primer (AP1: 5’-CCATCCTAAATACGACCTATAGGGC-3’). The GSPs were designed based on the conserved DNA sequences obtained from F. gigantica cathepsin L1 (GenBank accession number AF112566), cathepsin L1-B (GenBank accession number AF239264), and cathepsin L1-E (GenBank accession number AF239267) mature enzyme. The GSP1 (antisense primer) for the 5’-RACE PCR was 5’-TCACGGGAATCTGTCATCGGAGAC-3’ and the GSP2 (sense primer) for the 3’-RACE PCR was 5’-CCCGACAAAAATGTGCCGCTGAAATCCTG-3’.

Cloning of cDNA and DNA sequencing. The 5’-RACE PCR product was selected based on the sequence of the full-length cathepsin L1 and cloned into pGEM™-T Easy vector (Promega). Electrottransformation of E. coli JM 109 high efficiency competent cells was performed according to the protocol as previously described. White bacterial colonies were randomly selected and plasmids containing inserts were purified and sequenced. The nucleotide sequence of the gene was sequenced in both directions by the dye terminalsequencing method using Dye Terminator Cycle Sequencing kits (Applied Biosystems, Foster City, CA) and an ABI DNA sequencer 373A (Applied Biosystems). The BLAST network service was used to search the DNA and protein databases of the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov). The alignment of the sequences was carried out using the Multalin program (http://prodes.toulouse.inra.fr/multalin/multalin.html). The plasmids containing inserts were digested with Eco RI. The inserts were purified and subcloned into pCAL-n-FLAG expression vectors (Stratagene, La Jolla, CA) that were previously digested with Eco RI. The resulting plasmids were transformed into E. coli BL21 gold (DE3; Stratagene). The transformed cells were then examined and the existence of an insert was confirmed by a PCR and sequencing.

Expression and purification of the expressed protein. The expression of calmodulin binding peptide fused with cathepsin L-like in transformed cells was induced with 1 mM isopropyl-β-D-thiogalactopyranoside for three hours at 30°C. The cells were then harvested and the cell pellet was resuspended in cooled 0.01 M phosphate-buffered saline (PBS), pH 7.4, containing 0.1% Triton X and 1% sarcosine. The cells were then sonicated and the resulting suspension was centrifuged at 15,000 × g for 10 minutes at 4°C. Recombinant protein expressed as inclusion bodies was obtained from the pellet. The pellet was then washed several times with 0.01 M PBS, pH 7.4, and resuspended in solubilizing solution (50 mM Tris, pH 7.4, containing 0.1% Triton X and 1% sarcosine). The cells were then sonicated and the resulting suspension was centrifuged at 15,000 × g for 30 minutes. After centrifugation at 15,000 × g for 30 minutes, the first supernatant fraction (S1) was collected. The remaining pellet was repeatedly solubilized and centrifuged two times. The second (S2) and third (S3) supernatant fractions as well as the final remaining pellet were also collected. The S1, S2, S3, and pellet were then analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting. The S2 and S3 were selected for use as antigen for the cystatin capture ELISA.

Protein determination. The protein concentration was determined by the method of Bradford15 with bovine serum albumin (BSA) used as the standard.

Analytical SDS-PAGE and immunoblotting. The tested samples were analyzed by SDS-PAGE using the Mini-Protein II Cell (Bio-Rad Laboratories, Hercules, CA), under reducing conditions on a 10–18% polyacrylamide gradient gel prepared by the method of Laemmli. After electrophoresis, the resolved polypeptides were electrophoretically transferred to a nitrocellulose membrane for immunoblotting.17 For immunoblotting, the blotted membrane was blocked with a blocking solution (100 mM PBS, pH 7.5, 0.1% Tween 20, 1% skim milk) for 30 minutes at room temperature, followed by incubation with each serum samples (diluted 1:100 in blocking solution) for two hours. After washing with blocking buffer, the membrane was incubated with 1:1000 dilution of secondary, peroxidase-conjugated goat anti-human immunoglobulin G (Cappel; ICN Pharmaceuticals, Inc., Aurora, OH) and detected by chemiluminescence.
Preparation of recombinant protein antigen for the cystatin capture ELISA. The S2 and S3 fractions containing rCTL1 were pooled, dialyzed, lyophilized, and resuspended in 1% SDS at a final concentration of 2 mg/mL. The resulting suspension was stored at 4°C until used.

Cystatin capture ELISA. The method was performed as previously described18 with some modifications. Each well of the ELISA plate was sensitized with 1 μg of chicken egg cystatin (Sigma, St. Louis, MO) in 0.1 mL of 0.1 M carbonate buffer, pH 9.6, overnight at 4°C. The wells were washed five times with 10 mM PBS, pH 7.4, 0.05% Tween 20 (PBS/T) and blocked with 2% BSA in PBS/T for one hour at room temperature. After washing with PBS/T, 3 μg of recombinant protein antigen diluted with 1% BSA in PBS/T was added to the well and the plate was incubated overnight at 4°C. After another washing step with PBS/T, the wells were incubated for one hour at 37°C with 0.1 mL of human sera diluted 1:200 with 1% BSA in PBS/T. After washing with PBS/T, peroxidase-conjugated anti-human IgG (Cappel; ICN Pharmaceuticals, Inc.) diluted 1:40,000 with 1% BSA in PBS/T was used as secondary antibody. The wells were then washed with PBS/T and incubated with the 0.1 mL of o-phenylenediamine dihydrochloride substrate for 30 minutes. The reaction was stopped with 0.05 mL of 8 N H2SO4 and the absorbance was measured at 492 nm using a microplate ELISA reader (Tecan, Salzburg, Austria).

Statistical analysis. Statistical analysis was performed with the Student’s t-test using Sigma Stat (San Rafael, CA) statistical software. The diagnostic sensitivity, specificity, accuracy, positive predictive value, and negative predictive value were calculated and expressed as follows: sensitivity was the [number of true positives/(number of true positives + number of false negatives)] × 100; specificity was the [number of true negatives/(number of false positives + number of true negatives)] × 100; accuracy was the [number of true positives + number of true negatives/(number of true positives + number of false negatives + number of false positives + number of true negatives)] × 100; positive predictive value was the [number of true positives/(number of false positives + number of true positives)] × 100; negative predictive value was the [number of true negatives/(number of false negatives + number of true negatives)] × 100; true negative was the number of control samples (other parasitosis, cholangiocarcinoma, and healthy controls) that were negative by the assay; true positive was the number of proven fascioliasis samples that were positive by the assay; false positive was the number of control samples that were positive by the assay; false negative was the number of proven fascioliasis samples that were negative by the assay.

RESULTS

Sequence analysis of the cathepsin L1 gene. Following the RACE PCR, we obtained the full-length cathepsin L1 gene of the F. gigantica adult worm. An analysis of the deduced amino acid sequence of the selected subcloned PCR product showed a homology level of 99.0% compared with the F. gigantica cathepsin L1 sequence (GenBank accession number AF112566) with only two differences at position 123 from leucine to valine and position 212 from valine to alanine. The expression of the cathepsin L1 gene inserted into the pCAL-n-FLAG expression vector in E. coli resulted in an expressed fusion protein with an addition of a 46-amino acid fusion on the N-terminus, which contained a calmodulin binding protein tag (MKRRWKNNFIAVSAANRFKKISSSGALLVPR-GSDYKDDDDKGRGSE) and an enterokinase cleavage site. The recombinant protein was expressed in the inclusion body. Cells were then lysed under denaturing conditions using urea as the denaturant. The SDS-PAGE and immunoblot analysis of the expressed protein showed a protein with a molecular mass of approximately 35 kD. The expressed protein reacted strongly with the pooled positive reference sera, but not with the pooled negative reference sera (Figure 1).

Diagnostic values of the cystatin capture ELISA. The pooled S2 and S3 fractions containing rCTL1 were used as antigen in the cystatin capture ELISA for the detection of IgG antibody in the sera of patients with proven fascioliasis. The results were compared with those obtained using sera from patients with parasitic diseases other than fascioliasis, those with cholangiocarcinoma, and from healthy controls (Figure 2). The mean ± SD and ranges of optical densities (ODs) at 492 nm of the four sera groups are shown in Table 1. There was a significant difference in mean OD values between cases of proven fascioliasis (group 1) and cases of parasitic diseases other than fascioliasis (group 2), cholangiocarcinoma (group 3), and healthy controls (group 4) (P < 0.001). If the absorbance value of 0.464 (which was equivalent to the absorbance value of the mean plus 3 SD of the healthy control group) was used as the cut-off limit between positivity and negativity for fascioliasis, the sensitivity, specificity, accuracy, positive predictive value, and negative predictive value were 100%, 98.92%, 98.97%, 81.25%, and 100%, respectively. However, two cases of paragonimiasis and one case of gnathostomiasis showed false-positive results.

DISCUSSION

Serologic tests for the diagnosis of fascioliasis have been developed as standard assays using authentic F. hepatica ca-
thepsin L1 or rCTL1 as marker antigens. In the present study, the cDNA-encoded mature cathepsin L1 was expressed in E. coli. The expressed protein aggregated in the cytoplasm of E. coli and was solubilized only with strong denaturants such as urea. Therefore, investigation of the precise role of the protease was not performed. However, we have developed a diagnostic catstatin capture ELISA to detect antibodies to F. gigantica in humans that is based on the use of an rCTL1 protein as the marker antigen.

This study used chicken cystatin, which has a very low Kᵢ value and high specificity for cysteine protease, as a capture reagent for rCTL1 in the ELISA. This method could detect the antibodies to proteases without prior purification of rCTL1 from the bacterial lysate. This represents a big improvement in time, labor, and costs because purification of the recombinant protein requires specialized equipment and procedures, and it is troublesome to prepare rCTL1 because it requires several purification steps.

The catstatin capture ELISA is a sensitive and specific tool for the serodiagnosis of human fascioliasis. The procedure is simple and easy for use. The sensitivity was the same as for the ELISA using authentic FG27 antigen for the detection of human fascioliasis, while the specificity of rCTL1 was slightly lower (1.08%) than the one observed with the authentic FG27 antigen. Cross-reactions with sera from two cases of paragonimiasis and gnathostomiasis were demonstrated, which did not react with authentic FG27 antigen. This could either be due to a subclinical infection with Fasciola spp. or because the rCTL1 shares some epitope with paragonimiasis and gnathostomiasis. However, the another important element of this study was the ability to demonstrate that, contrary to FG27, the rCTL1 antigen could be produced in large amounts to produce a standardized serological test for human fascioliasis.

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Authors’ addresses: Chairat Tantrawatapan, Chaisiri Wongkham, and Sopit Wongkham, Department of Biochemistry, Faculty of Medicine, Khon Kaen University, Khon Kaen 40002, Thailand. Wanchai Maleewong and Pewpan M. Intapan, Department of Parasitology, Faculty of Medicine, Khon Kaen University, Khon Kaen 40002, Thailand. Kunio Nakashima, Faculty of Human Health, Tokai Gakuen University, Nakahira 2-901, Tenpaku-ku, Nagoya, Aichi, Japan.

Reprint requests: Wanchai Maleewong, Department of Parasitology, Faculty of Medicine, Khon Kaen University, Khon Kaen 40002, Thailand. Telephone: 66-43-348387, Fax: 66-43-202475, E-mail: wanch_ma@kku.ac.th.

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